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Development and optimization of a method for analyzing biodiesel mixtures with non aqueous reversed phase liquid chromatography

G. Di Nicola, M. Pacetti, F. Polonara, G. Santori and R. Stryjek

Abstract

Biodiesel (a mixture of fatty acid esters) is normally analyzed using gas chromatography/flame ionization detection, as specified by the ASTM D6584 and EN14105 standards. This paper proposes a binary gradient method for analyzing biodiesel mixtures using non-aqueous reverse phase HPLC with a UV detector capable of overcoming the drawbacks of the gas chromatographic technique normally used. The new analytical method was developed by means of a statistical sensitivity analysis applied to the main parameters influencing the recording, using the full factorial design method combined with the Yates algorithm and the steepest ascent optimization procedure. The present study shows the influence of the main biodiesel mixture separation analysis parameters. The resulting tool proved valid for analyzing not only biodiesel, but also any traces of unreacted oil.

1. Introduction

In many countries, biodiesel is now considered as an alternative to conventional fossil fuels. It is increasingly frequently mixed with commercial diesel, even in considerable quantities, because of the advantage it offers in terms of economic convenience and environmental sustainability [1, 2]. Biodiesel is the product of the transesterification of vegetable oils (typically rape seed, sunflower seed, palm and soya bean oils) and is consequently renewable and readily obtainable. The main constituents of vegetable oils are triacylglycerols (TGs) [3]. The transesterification reaction of these TGs with alcohol gives rise to fatty acid esters, commonly known as biodiesel and 1,2,3 propanetriol (glycerol) [4]. Current specifications only use the term biodiesel to define mixtures of compounds containing no more than 0.3% of alcohol, 0.8% of monoacylglycerols (MGs), 0.4% of diacylglycerols (DGs), 0.4% of triacylglycerols, 0.02% of free glycerol (GL), and 0.25% of total glycerol [5], so an accurate analysis of the complex mix of substances taking part in the production reaction is fundamental [6]. Transesterification is an equilibrium reaction that can be achieved with an alkaline, acid or enzymatic catalyst, as explained in [7, 8]. The system that has met with the greatest success uses methanol as the alcohol and KOH as the alkaline catalyst [4, 9-12]. Schematically, the global reaction proceeds as follows:

$$Triacylglycerol + 3R'OH \leftrightarrow Glycerol + 3R'COOR$$

The intermediate steps in the reaction are:

$$Triacylglycerol + R'OH \leftrightarrow Diacylglicerol + R'COOR_1$$
$$Diacylglycerols + R'OH \leftrightarrow Monoacylglycerol + R'COOR_2$$
$$Monoacylglycerol + R'OH \leftrightarrow Glycerol + R'COOR_3$$

There are three consecutive reactions giving rise to the formation of DGs, MGs, three moles of fatty acid esters and one mole of GL. Numerous analytical methods are suitable for characterizing a mixture composed of reactants and products of transesterification [13-15]. The method that has been most successful is the one recommended in the ASTM standard D6584 and its European counterpart, EN 14105 [5], which provide specifications on the use of gas chromatography and an flame ionization detector. This analytical method is not very convenient for identifying non-volatile triacylglycerols, however [10]: these compounds need to be derivatized and analyzed at high temperatures (approximately 350°C), with a consequent fallout on the life of the column. To avoid the derivatization step and enable even triacylglycerols with a higher molecular mass to be recorded, high performance liquid chromatography (HPLC) methods have been developed that allow for the sample to be kept in the liquid state, without any pretreatment. As reported in [3, 7, 16-20], various detectors have been used in combination with HPLC, e.g. UV detectors, refractive index detectors, evaporative light scattering detectors, or the more expensive and more suitable mass spectrometer detectors,

but the most efficient methods use the gradient chromatographic process because this affords great flexibility in the analytical setup and thus enables a variety of substances to be measured. One of the most common and valuable HPLC techniques [21] is based on a linear ternary gradient consisting of two steps, the first aqueous-organic, the second non-aqueous. The proposed analysis consists of the following: 70% acetonitrile + 30% water at 0 min, 100% acetonitrile at 10 min, 50% acetonitrile + 50% isopropanol-hexane (5:4, v/v) from 20 to 25 min. A UV detector is used at 205nm. Using the recommended settings, MGs, fatty acid methyl esters (FAMEs), DGs and TGs are accurately distinguished and some of the main individual TGs can also be separated within the Equivalent Carbon Number (ECN) groups. The ECN is defined as ECN=CN-2DB, where CN is the number of carbon atoms and DB the number of double bonds, and it is a parameter for classifying fatty compounds. The present paper describes a method statistically optimized according to [22] that uses a binary non-aqueous gradient and a UV detector, achieving a separation efficiency comparable with the state of the art of biodiesel mixture analysis [23, 24], with the advantage of not expansive apparatus, set up in accordance with the optimal parameters for analyzing these compounds. The statistical analysis enables the main parameters influencing the separation to be quantified. The main compounds in the reacting mixture are also identified. The use of a non-aqueous rather than aqueous gradient enables the elution of less polar compounds, such as TGs, in relatively short analytical times. Although the method developed is bound to be influenced by the type of column used [25], the approach adopted and the results obtained provide information on the sensitivity of the compounds in different chromatographic conditions for their separation.

2. Experimental

2.1. *Reagents and materials*

All solvents were HPLC grade and were used without purification. They included: Sigma-Aldrich (Milan, Italy) methanol Chromasolv Plus for HPLC ≥ 99.9%, acetonitrile G Chromasolv for HPLC super gradient grade, n-hexane Chromasolv for HPLC, and isopropanol G Chromasolv. A Teknokroma (Barcelona, Spain) Mediterranea sea18 analytical column, internal diameter 4.6 mm, length 25 cm, particle size 5µm, was used for the analyses. Commercial edible-grade sunflower oil and Fluka (Milan, Italy) methanol 99.8% were purchased for transesterification. The reference standards were obtained from Nu-Chek-Prep., Inc. (Elysian, Minnesota-USA) and coded as TLC-18-3A (1,3 monolinolenin, 1,2 monolinolenin, methyl-ester of linolenic acid, 1,2 dilinolenin, 1,3 dilinolenin, trilinolenin), TLC-18-2A (1,3 monolinolein, 1,2 monolinolein, methyl-ester of linoleic acid, 1,2 dilinolein, 1,3 dilinolein, trilinolein), TLC-18-1A (1,3 monolein, 1,2 monolein, methyl-ester of oleic acid, 1,2 diolein, 1,3 diolein, triolein), TLC-18-0A (1,3 monostearin, 1,2 monostearin, methyl-ester of stearic acid, 1,2 distearin, 1,3 distearin, tristearin), TLC-16-0A (1,3 monopalmitin, 1,2 monopalmitin, methyl-ester of palmitic acid, 1,2 dipalmitin, 1,3 dipalmitin, tripalmitin). Table 1 lists the abbreviations used to represent the acylic chains.

Table 1
Abbreviations for acyl radicals

Trivial name	Systematic name	Symbol	CN:DB	ECN
Palmitoyl	Hexadecanoyl	P	C16:0	16
Stearoyl	Octadecanoyl	S	C18:0	18
Oleoyl	Octadecenoyl	O	C18:1	16
Linoleoyl	Octadecadienoyl	L	C18:2	14
Linolenoyl	Octadecatrienoyl	Ln	C18:3	12

2.2. *Chromatographic instrumentation*

The analyses were conducted with a Shimadzu VP series HPLC equipped with two LC-10ADVP solvent delivery units for binary gradient elution, a model SPD-10AVVP UV-vis detector, a model RID10A differential refractometer, a manual Rheodyne 7725(i) sample injector, a model CTO-10ASVP columns oven for precision temperature control at sub-ambient temperatures too, a model SCL-10AVP system controller, and LC-Solution 2.1 software for remote management.

2.3. *Sample preparation*

The transesterification reaction was obtained on sunflower seed oil with methanol in proportions of 6:1 using KOH for approximately 30 minutes to obtain a mixture of methyl esters and unreacted oil at a temperature of 45°C [7]. The resulting mixture after filtration was used to prepare the samples for the tests. One sample was dissolved in hexane-isopropanol 3:5 v/v and another was dissolved in hexane-isopropanol

6:5 v/v, always obtaining a sample-to-solvent ratio of 1:30 (w/w). This procedure was used in accordance with the rule that the sample must be diluted in the solvent used for elution. Then the tests required for the statistical analysis were conducted, setting the parameters each time according to the demands of the full factorial design (FFD) method [26-28]. The constant parameters for the analysis were the temperature of the thermostatically-controlled compartment of the column, set at 30°C to avoid methanol evaporation problems, and the wavelength of the UV detector, set at 210 nm to ensure the transparency of the mixtures used as solvents without exceeding 220 nm, a value nearing the wavelength limit for the analysis FAMEs and glycerides. In fact, the UV spectrum of a mixture of FAMEs declines beyond around 220 nm [29]. For the analysis of the standards in the liquid phase, TLC 18 3A, TLC 18 2A and TLC 18 1A, solutions of 1:50 (w/w) were prepared in n-hexane-isopropanol 8:5 (v/v), then diluted to obtain solutions of 1:60 (w/w), 1:70 (w/w), 1:80 (w/w), 1:100 (w/w). For the standards in the solid phase, TLC 18 0A and TLC 16 0A, solutions of 1:100 (w/w) were prepared in n-hexane-isopropanol 8:5 (v/v), then diluted to 1:120 (w/w), 1:140 (w/w), 1:150 (w/w). The higher dilution ratio of the standards for the samples used in the statistical analysis was justified by the need to identify only the main compounds involved in transesterification.

2.4. *Optimization strategy*

The object of the present study was to develop a chromatographic method for analyzing mixtures containing esters and glycerides by studying the sensitivity of the compounds under analysis to the selected chromatographic parameters. This demands a preliminary investigation to find the optimal configuration for ensuring the maximum separation of the compounds, recording the maximum number of compounds and the minimum duration of the analysis. First of all, the FFD method was used on two levels to find the factors that most affect the analysis, then the steepest ascent method (SAM) was used to identify the configuration that produces the best chromatogram. The condition studied includes five parameters, giving rise to a 2^5 factorial design. The most relevant parameters examined were: *A*, total flow rate (*tot* ml/min); *B*, gradient start time (*t1* min); *C*, gradient end time (*t2* min); *D*, percentage of mobile phase A (acetonitrile/methanol 4:1 v/v) in the second part of the elution (*x%*); *E*, mixing proportion between n-hexane and isopropanol in the mobile phase B (*f* v/v). The two levels of variation of the parameters are given in Table 2. The effects observed were the number of peaks recorded (*n*), the mean resolution of the peaks recorded (*Rs*) and the retention time (*RT*), setting a limit of 40 min after inserting the sample to arrive at an optimized method that does not take too long. The optimization was done to find the conditions that maximize the *y* result function:

$$y = \frac{\sum_{i=1}^{n} Rs_i}{n-1} n$$

Table 2
Factors and levels used for full factorial design

Parameters		Low value (-)	Central value (0)	High value (+)
A	Total flow rate *(tot)*	0.7 ml/min	0.85 ml/min	1 ml/min
B	Linear gradient start time *(t1)*	5 min	7.5 min	10 min
C	Linear gradient end time *(t2)*	15 min	17.5 min	20 min
D	Percentage of mobile phase A *(x%)*	30%	45%	60%
E	Mixing proportion mobile phase B *(f)*	0.6 (3:5 v/v)	0.9	1.2 (6:5 v/v)

where Rs_i is the resolution of the i-th peak by comparison with its predecessor, and *n* is the number of peaks recorded. By adopting the objective function, *y*, and stopping the acquisitions after 40 min, we aimed to obtain a method with a predilection not only for the mean resolution - as a significant factor for optimizing the method - but also for the capacity for separating the various compounds in the complex mixture being analyzed within a time span consistent with the purpose of the analysis.

3. Results and discussion

In chromatography, when a method needs to be optimized, the most commonly-adopted strategy is the one called One Factor At a Time (OFAT), but this strategy does not enable an effective optimization of the analytical conditions, and thus gives rise to methods that are not always ideal. Drawing inspiration from other works [30, 31], we applied the FFD method to determine the parameters most influential on the chromatographic method presented herein. Said parameters were then raised or lowered using the SAM

procedure [32, 33], which not only enabled us to pinpoint the chromatographic conditions nearest to the optimal configuration, but also implicitly assessed the robustness of the method.

3.1. *Experimental design*

The full two-level (2^5) factorial experimental design meant planning series of experiments with changing variables, as shown in the design matrix in Table 3. We obtained 32 chromatograms from unreplicated experiments. For each experiment, we considered the number of peaks recorded, the duration of the chromatogram considered as the retention time of the last peak recorded and the function result *y*. The effects of the single variables and of the higher-order interactions were estimated using the Yates algorithm [22]. The single parameters and their interactions influence the result presented in Table 3. Classical statistical tools, such as Student's distribution, were applied to identify significant factors and interactions. Considering the third-, fourth- and fifth-order interactions, a Student's t distribution with 16 degrees of freedom is needed. Using a 95% confidence interval for the established Student's distribution, two-level interactions between the variables *A*, *D*, *E* also revealed a significant influence on responses because the value of their effect declines outside said confidence interval. The combined effects *DE*, *AD*, *ADE* are more important than the single effects *A*, *D*, *E*. The effects and significance of the variables are shown in Figure 1 for the response *y*. For the second- and third-order effects, two-way tables were obtained for the effects *AD* and *DE*, and the three-way table for the effect ADE. This procedure enables a better assessment of how the result responds to variations in the parameters involved. The combined study considers the mean of the results obtained from the tests using the parameters set as stated. Figure 2 shows that a synergic effect can be obtained when the factors *tot* (*A*), *x* (*D*) and *f* (*E*) are moved in the positive direction.The order of significance of the main parameters is *A*>*C*>*E*>*D*>*B* for *y*. The sensitivity of the result depends primarily on the flow rate of the mobile phase. The results obtained confirm the gradient theory [34], i.e. the increase in mobile phase volume passing through the column influences the retention time rather than the gradient time.

Table 3

Design matrix, results of factorial experiments and Yates analysis

Run	tot	t1	t2	x	f	n	RT[a]	$\frac{\sum_{i=1}^{n} Rs_i}{n-1}$	y	I	II	III	IV	V	Effects	ID[b]
1	-	-	-	-	-	39	29.21	1.36	53.19	117.23	236.89	499.61	973.64	2011.99	62.87	*Mean*
2	+	-	-	-	-	34	24.25	1.88	64.04	119.66	262.71	474.03	1038.36	187.36	11.71	*A*
3	-	+	-	-	-	30	30.31	1.93	57.97	129.38	225.77	484.10	103.15	-43.36	-2.71	*B*
4	+	+	-	-	-	33	25.17	1.87	61.70	133.33	248.26	554.25	84.21	-7.82	-0.49	*AB*
5	-	-	+	-	-	40	32.57	1.52	60.89	118.90	231.27	26.60	-22.25	85.60	5.35	*C*
6	+	-	+	-	-	43	28.14	1.59	68.49	106.87	252.83	76.55	-21.11	-0.60	-0.04	*AC*
7	-	+	+	-	-	38	34.14	1.70	64.45	132.43	269.26	42.94	-3.47	-1.71	-0.11	*BC*
8	+	+	+	-	-	37	29.15	1.86	68.87	115.83	284.99	41.27	-4.36	13.69	0.86	*ABC*
9	-	-	-	+	-	32	33.48	1.51	48.41	120.63	14.58	6.37	48.30	44.58	2.79	*D*
10	+	-	-	+	-	29	34.12	2.43	70.49	110.63	12.02	-28.63	37.30	48.27	3.02	*AD*
11	-	+	-	+	-	31	34.78	1.46	45.31	128.99	38.34	-15.15	-2.69	-25.81	-1.61	*BD*
12	+	+	-	+	-	26	30.86	2.37	61.57	123.84	38.21	-5.96	2.09	6.20	0.39	*ABD*
13	-	-	+	+	-	32	37.00	1.87	59.82	135.24	22.74	-10.30	-3.05	-9.17	-0.57	*CD*
14	+	-	+	+	-	30	37.70	2.42	72.60	134.02	20.20	6.84	1.34	9.62	0.60	*ACD*
15	-	+	+	+	-	30	37.08	1.51	45.20	144.86	18.32	3.29	22.41	-14.43	-0.90	*BCD*
16	+	+	+	+	-	26	33.85	2.72	70.63	140.13	22.95	-7.65	-8.72	4.77	0.30	*ABCD*
17	-	-	-	-	+	37	26.90	1.49	54.98	10.85	2.43	25.82	-25.58	64.72	4.04	*E*
18	+	-	-	-	+	37	25.97	1.77	65.66	3.73	3.94	22.48	70.15	-18.94	-1.18	*AE*
19	-	+	-	-	+	31	28.04	1.59	49.28	7.60	-12.03	21.57	49.94	1.14	0.07	*BE*
20	+	+	-	-	+	31	24.07	1.98	61.35	4.42	-16.60	15.73	-1.67	-0.89	-0.06	*ABE*
21	-	-	+	-	+	41	30.65	1.46	59.92	22.08	-10.00	-2.56	-35.00	-11.01	-0.69	*CE*
22	+	-	+	-	+	41	26.73	1.68	69.07	16.26	-5.15	-0.12	9.19	4.78	0.30	*ACE*
23	-	+	+	-	+	33	40.32	1.71	56.39	12.78	-1.23	-2.54	17.14	4.40	0.27	*BCE*
24	+	+	+	-	+	39	27.68	1.73	67.45	25.43	-4.73	4.63	-10.94	-31.13	-1.95	*ABCE*
25	-	-	-	+	+	32	37.30	1.98	63.24	10.68	-7.12	1.51	-3.33	95.73	5.98	*DE*
26	+	-	-	+	+	36	36.25	2.00	72.00	12.06	-3.18	-4.57	-5.83	-51.62	-3.23	*ADE*
27	-	+	-	+	+	33	36.18	1.89	62.23	9.15	-5.82	4.85	2.44	44.19	2.76	*BDE*
28	+	+	-	+	+	32	33.21	2.24	71.79	11.05	12.65	-3.51	7.18	-28.08	-1.76	*ABDE*
29	-	-	+	+	+	34	37.58	1.90	64.58	8.76	1.39	3.95	-6.08	-2.50	-0.16	*CDE*
30	+	-	+	+	+	34	35.26	2.36	80.28	9.56	1.90	18.47	-8.35	4.74	0.30	*ACDE*
31	-	+	+	+	+	32	38.57	2.08	66.44	15.70	0.79	0.52	14.52	-2.28	-0.14	*BCDE*
32	+	+	+	+	+	31	33.90	2.38	73.69	7.25	-8.44	-9.24	-9.75	-24.28	-1.52	*ABCDE*

[a]RT is the retention time of each compound;

[b]ID is the identification code of the parameter or of interaction between parameters;

See section 2.4 for the factors meaning

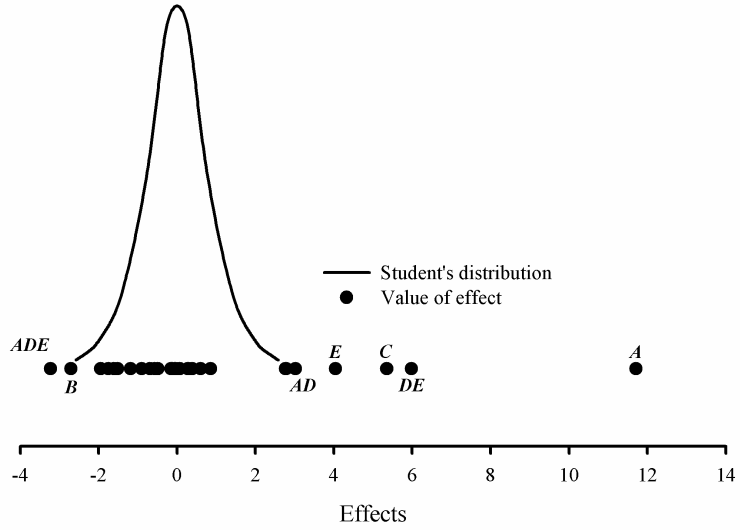


Fig. 1. Comparison between the effects calculated by the Yates algorithm and Student's distribution with 16 degrees of freedom, considering a 95% confidence interval to determine the main parameters and the interactions that most affect the gradient.

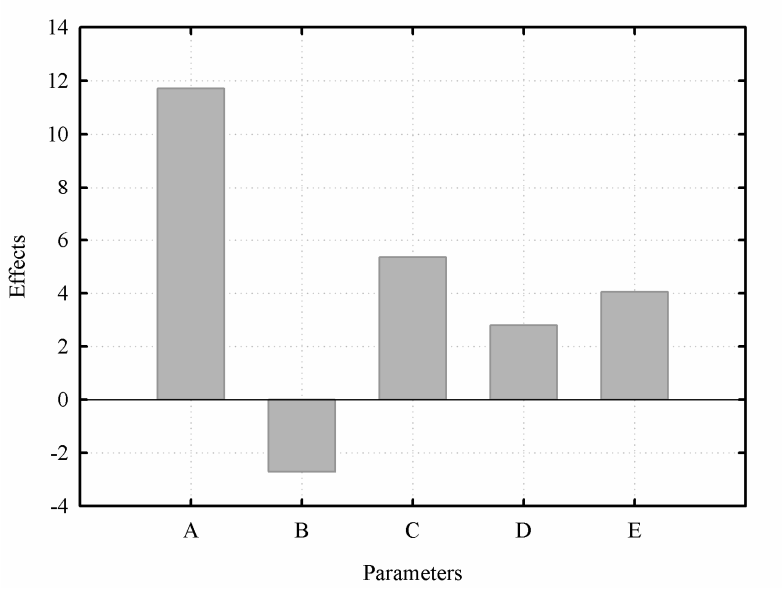


Fig. 2. Pareto chart of estimated effects of main parameters.

3.2. *Path of steepest ascent*

The results emerging from the factorial design clearly show that the optimal region was beyond the current design range. In this situation, a directional search method, such as SAM, can be used to identify the optimal conditions. The path of steepest ascent begins at the arbitrary point of the design space examined and continues beyond the design space, and an equally-spaced sequence of parameters is planned along said path. As shown in Figure 2, in the direction of steepest ascent (based on the main effect factors) the positive value of the main effects indicates that increases in parameters *A, C, D, E* and decreases in the quantity of factor *B* will increase the yield. The values obtained for the result *y* in these experiments are summarized in Table 4. We could have continued beyond the seven tests conducted, but this would have meant increasing the flow rate to more than 1.3 ml/min and, though it would have contributed a further improvement, this was considered excessive and inconsistent with the purpose of the present optimization. As shown in Table 4, an increase in the flow rate induces an increase in the gradient time too, as contemplated in the gradient theory [34].

Table 4
Normalized step calculation and results of steepest ascent experiments

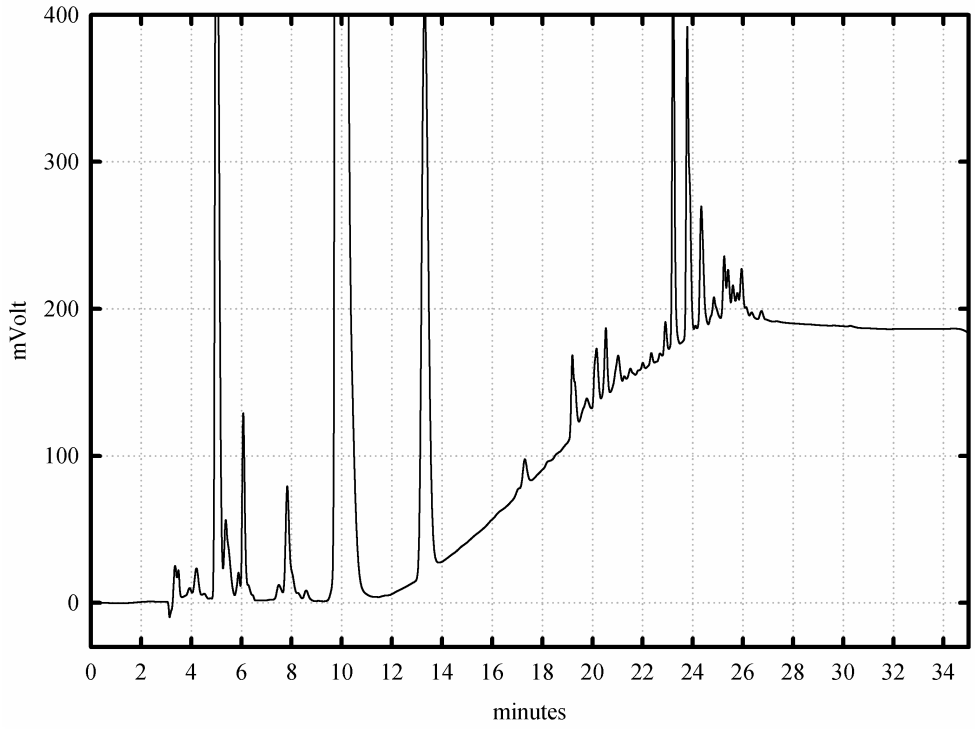
	Factors					Results
	tot (*A*)	t1 (*B*)	t2 (*C*)	x (*D*)	f (*E*)	
Initial level	0.80	7.50	17.50	0.40	0.80	
Increment	0.10	2.00	2.00	0.10	0.20	
Coefficient	11.71	-2.71	5.35	2.79	4.04	
Incr.* coeff.	1.17	-5.42	10.70	0.28	0.81	
Normalized step	0.10	-0.46	0.91	0.02	0.07	
Run 0	0.7	5.00	20.00	0.20	1.20	53.75
Run 1	0.8	4.54	20.91	0.22	1.27	71.47
Run 2	0.9	4.07	21.83	0.25	1.34	75.96
Run 3	1	3.61	22.74	0.27	1.41	77.03
Run 4	1.1	3.15	23.66	0.30	1.48	76.98
Run 5	1.2	2.69	24.57	0.32	1.55	77.32
Run 6	1.3	2.22	25.48	0.34	1.61	88.08

3.3 *Method development*

An optimized method was obtained for determining TGs, DGs, MGs and FAMEs in incompletely reacted mixtures. During transesterification, these compounds occur in variable quantities, depending on the kinetics of the reaction. The chromatographic method proposed in the present paper was developed to follow up this reaction. A two-level full factorial matrix was applied to screen significant factors for the elution. Low and high values were selected for the variables on the strength of the results of preliminary experiments, which showed that: (1) acetonitrile/methanol 4:1 (v/v) with isocratic elution is a suitable mobile phase for determining FAMEs because it demonstrated a good capacity for separating the substances relatively rapidly; (2) the hexane-isopropanol system with isocratic elution is a good mobile phase for separating acylglycerols; (3) the oven temperature was kept constant at 30°C because it must not come too close to the temperature at which methanol evaporates at ambient pressure; (4) the absorption spectrum of acetonitrile(ACN)/methanol(MeOH) 4:1 (v/v) shows that the minimum UV wavelength must be set at 210nm. Although the results of the FFD show a better resolution of the peaks when the percentage of mobile phase A in the second part of the elution *x* is set at higher values, the chromatograms obtained in these tests did not include the TGs, which could only be recorded by extending the acquisition time beyond 40 minutes. So the result *y* to optimize must take into account the mean resolution of the peaks and the number of peaks recorded, and the number of compounds identifiable in the mixture as a consequence. A high mean resolution coincides with a limited number of peaks because the compounds tend to be coeluted; vice versa, when the number of peaks is high, the resolution is low and the chromatogram takes longer than 40 min. This was true of all the test with *x*=60% where the chromatogram appears to be separated into several, well-spaced peak clusters [35], when we were unable to record the TGs within 40 min. In the subsequent optimization, the starting value of *x* was consequently set very low (20%) and in this case there is evidence of a considerable improvement in the separation of the DGs. The SAM was applied to optimize response when the process moved from the basal level to a point in the factor space where *y* was maximized. Step size was calculated from the main effects alone. The six SAM steps show a continual improvement in the chromatogram for the three aspects considered, i.e. mean resolution of the peaks, number of substances recorded, retention time of the last recorded peak. Table 5 summarizes the characteristics of the optimized method. The results obtained with mixtures derived from sunflower seed oil indicate that, for analyses of this type, we need to achieve a linear gradient on an RP C_{18} column as follows:mobile phase A (0 to 2.2 min), up to 34% mobile phase A 66% mobile phase B (25.5 to 30 min), with a flow rate of 1.3 ml/min. Figure 3 compares the chromatogram with the highest response obtained with the experimental design against the chromatogram obtained applying the optimized method.

Table 5
Optimal setup of the proposed method

Flow rate (ml/min)	Mobile phase A		Mobile phase B	
1.3	Acetonitrile: Methanol	4:1 (v/v)	n-Hexane: Isopropanol	8:5 (v/v)
Time (min)	Mobile phase			
0	Mobile phase A			
2.2	Mobile phase A			
25.5	Mobile phase A 34%- Mobile phase B 66%			
30	Mobile phase A 34%- Mobile phase B 66%			



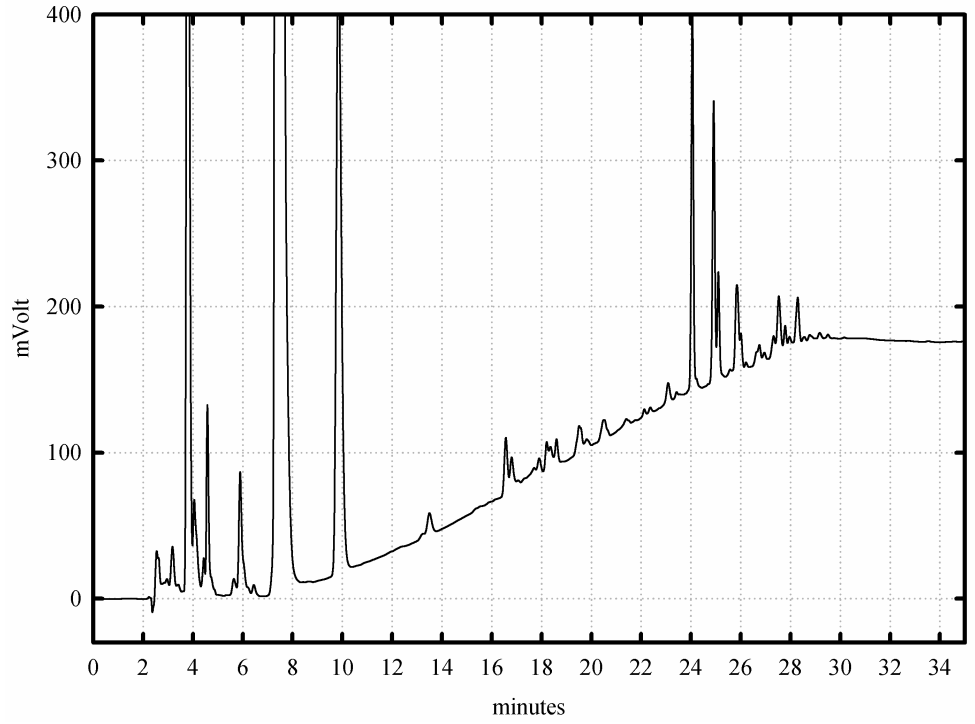


Fig. 3. Comparison between the best chromatogram obtained with the experimental design (above) and the chromatogram obtained by applying the optimized method (below)

3.4 *Identification*

In studying transesterification kinetics, it is fundamental to identify the chemical species contained in the actual mixture during the reaction. This is done by separately analyzing the pure chemical species. Analyzing sunflower seed oil provided us with information on the positions of DGs and TGs in the chromatogram, also considering the ECN of TGs and the most important fatty acids (FAs) in sunflower seed oil, which has a large proportion of trilinolein, the TG with the lowest ECN. DGs have retention times below the peak for trilinolein. A stock of sunflower seed oil that had been transesterified several times was used as a standard for identifying the FAMEs. Every single FAME can be identified for the main fatty acids in sunflower seed oil. A mixture of fatty acids was obtained by hydrolysis of the vegetable oil and individual MGs were identified, excluding the compounds already analyzed from the intermediate reacting mixture. Figure 4 shows the chemical species identified and the order of elution was FAs>MGs>FAMEs>DGs>TGs. This preliminary procedure helps to separate the chemical species, but identifying individual compounds involves analyzing the standards and dividing the peaks into families with the same ECN. The retention times for all the main individual compounds identified are given in Table 6. The sunflower seed oil also revealed traces of linolenic acid compounds.

Table 6
Retention times and molecular masses of the compounds in a reacting mixture identified by UV detector

Retention time (min)	Relative retention r[a]	Name	Notation	ECN[b]	Molecular mass
Fatty acids					
2.93	0.51	Linolenic acid	Ln acid	-	278.2
3.24	1	Linoleic acid	L acid	-	280.3
3.43	1.23	Oleic acid	O acid	-	282.3
Monoacylglycerols					
3.94	0.72	1-Monolinolenin[c]	1-Ln	12	352.3
4.09	0.79	2-Monolinolenin[c]	2-Ln	12	352.3
4.55	1	2-Monolinolein[c]	2-L	14	354.3
4.69	1.07	1-Monolinolein[c]	1-L	14	354.3
5.79	1.58	1-Monopalmitolein[c]	1-P	16	330.3
5.87	1.61	1-Monoolein[c]	1-O	16	356.3
6.20	1.77	2-Monoolein[c]	2-O	16	356.3
8.10	2.65	1-Monostearin[c]	1-S	18	358.3
Fatty acid methyl esters					
6.03	0.70	Methyl ester of linolenic acid[c]	MeLn	-	292.2
7.60	1.0	Methyl ester of linoleic acid[c]	MeL	-	294.3
10.06	1.47	Methyl ester of oleic acid[c]	MeO	-	296.3
10.15	1.49	Methyl ester of palmitic acid[c]	MeP	-	270.3
13.73	2.18	Methyl ester of stearic acid[c]	MeS	-	298.3
Diacylglycerols					
13.11	0.75	1,3-Dilinolenin[c]	1,3-LnLn	24	612.5
13.65	0.79	1,2-Dilinolenin[c]	1,2-LnLn	24	612.5
16.61	1	1,3-Dilinolein[c]	1,3-LL	28	616.5
16.87	1.02	1,2-Dilinolein[c]	1,2-LL	28	616.5
17.99	1.10	1,3-Oleoyl-linoleoyl-glycerol	1,3-OL	30	618.5
18.19	1.11	1,2-Oleoyl-linoleoyl-glycerol	1,2-OL	30	618.5
18.28	1.12	1,3-Palmitoyl-linoleoyl-glycerol	1,3-PL	30	592.5
18.54	1.14	1,2-Palmitoyl-linoleoyl-glycerol	1,2-PL	30	592.5
19.71	1.22	1,3-Diolein[c]	1,3-OO	32	620.5
19.79	1.22	1,2-Diolein[c]	1,2-OO	32	620.5
20.02	1.24	1,3-Dipalmitin[c]	1,3-PP	32	568.9
20.22	1.25	1,2-Dipalmitin[c]	1,2-PP	32	568.9
20.35	1.26	1,3-Stearoyl-linoleoyl-glycerol	1,3-SL	32	620.5
20.41	1.27	1,2-Stearoyl-linoleoyl-glycerol	1,2-SL	32	620.5
20.77	1.29	1,3-Palmitoyl-oleoyl-glycerol	1,3-PO	32	594.5
21.04	1.31	1,2-Palmitoyl-oleoyl-glycerol	1,2-PO	32	594.5
21.16	1.32	1,3-Stearoyl-oleoyl-glycerol	1,3-SO	34	622.6
21.25	1.33	1,2-Stearoyl-oleoyl-glycerol	1,2-SO	34	622.6
22.36	1.40	1,3+1,2-Distearin[c]	1,3+1,2-SS	36	625.0
Triacylglycerols					
21.39	0.88	Trilinolenin[c]	LnLnLn	36	872.7
24.10	1	Trilinolein[c]	LLL	42	878.8
25.00	1.04	Oleoyl-dilinoleoyl-glycerol	OLL	44	880.8
25.19	1.05	Palmitoyl-dilinoleoyl-glycerol	PLL	44	854.8
25.94	1.08	Dioleoyl-linoleoyl-glycerol	OOL	46	882.8
26.08	1.09	Stearoyl-dilinoleoyl-glycerol	SLL	46	882.8
26.27	1.10	Palmitoyl-oleoyl-linoleoyl-glycerol	POL	46	856.8
26.53	1.11	Triolein[c]	OOO	48	884.8
26.74	1.12	Stearoyl-oleoyl-linoleoyl-glycerol	SOL	48	884.8
26.88	1.13	Palmitoyl-dioleoyl-glycerol	POO	48	858.8
27.09	1.14	Tripalmitin[c]	PPP	48	807.3
28.77	1.22	Tristearin[c]	SSS	54	891.5

[a] Relative retention $r=t_R'/t_S-t_M$, where $t_R'=t_R-t_M$ is the adjusted retention time with t_M=hold up time (2.4 min), t_S are retention times for a reference compound in each chemical species, t_R are retention times for each compound.
[b] ECN=CN-2*DB, where CN is the carbon number in all acyl chains and DB is the number of double bonds
[c] Reference standard

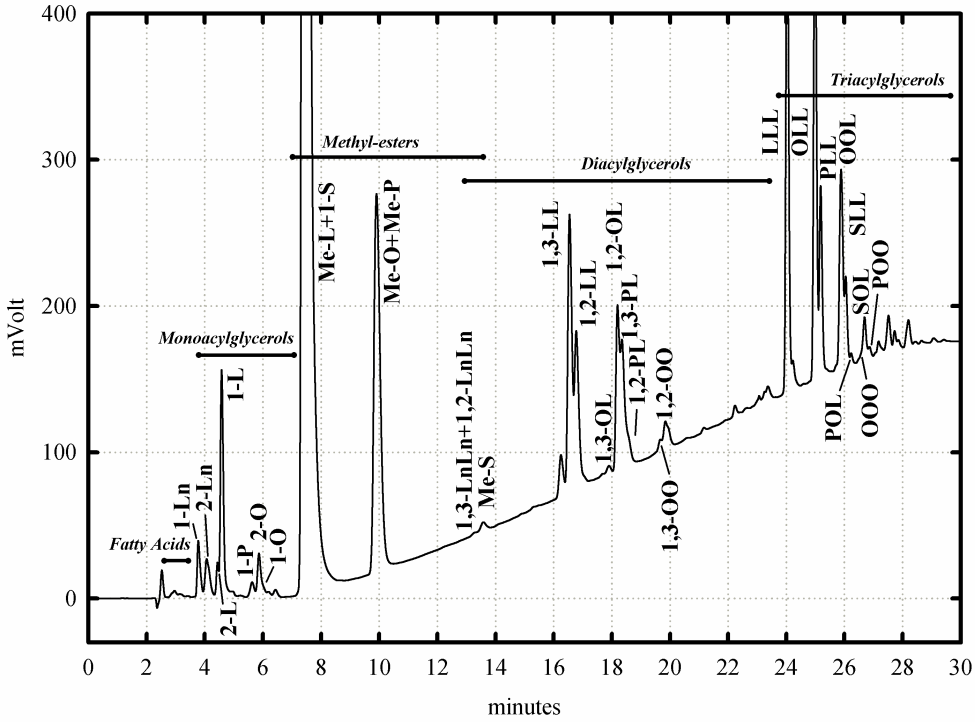


Fig. 4. Identification of chemical species and single compounds in a reacting mixture. For symbols see Table 1.

4. Conclusions

We developed a strategy for optimizing an RP-HPLC method for analyzing biodiesel mixtures, which consisted of preliminary experiments, a series of tests conducted using the full two-level factorial design statistical technique, and optimization steps as indicated in the SAM procedure. The method is based on the use of a fast and efficient chromatographic linear elution suitable for analyzing biodiesel and its related substances. Full factorial design showed that flow rate and gradient end time are the parameters most strongly affecting compound detection. The optimized conditions led to the robust identification with a high resolution of all the biodiesel mixture's components in less than 30 min. The qualitative analysis was conducted to classify each compound in the reacting mixture, starting from sunflower seed oil and from rapeseed oil. The results are consistent with data in the literature [23].

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